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Patent- og Varemærkestyrelsen
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PATENT- OG VAREMÆRKESTYRELSEN

TITLE: METHOD FOR TESTING OR SCREENING FOR AN ENZYME OF INTEREST

FIELD OF THE INVENTION

The present invention relates to methods for testing or screening host cells cultivated on or in a solid media for expression of enzymatic activity.

BACKGROUND OF THE INVENTION

Host cells are generally cultured in a liquid media or on a solid media, e.g. an agar plate.

When host cells are cultured on a solid media assays for identification of host cells which express a particular enzymatic activity generally utilise a substrate for the enzyme which is capable of changing colour upon reaction with the enzyme or they utilise a substrate labelled with a dye which is released upon reaction between the substrate and the enzyme. Typically the reaction between the substrate and the enzyme results in the formation of a halo with a different colour than the rest of the solid media surrounding those host cells/colony of host cells expressing the particular enzyme, thereby making it possible to distinguish host cells expressing the enzyme from those not expressing the enzyme.

For example skim milk is often added to the solid media for detection of protease activity as proteases are capable of cleaving the proteins in the white skim milk and thereby make the skim milk colourless (i.e. host cells expressing the protease activity are detected by the presence of a colour-less halo surrounding them, also known as clearing zones).

Another example is detection of alpha-galactosidase by the release of p-nitrophenol (which is yellow) from p-nitrophenol-alpha-galactosidase.

However, to increase the reliability and speed of the screening process for enzymatic activities there is a continuous need for improved assays for testing or screening for expression of enzyme activities by host cells cultured on solid media.

Ligninolytic enzyme production by *Polyporaceae* from Lombok, Indonesia is disclosed by Risna RA and Suhirman (2002), Fungal Diversity, 9, 123-134.

WO 93/11249 discloses a method of screening for a DNA sequence coding for a protein of interest.

SUMMARY OF THE INVENTION

The invention provides a method for testing or screening a host cell for expression of an enzyme of interest, comprising testing or screening for a change in the colour of a first dye by the presence of a host cell expressing the enzyme of interest, wherein the host cell has been cultivated on or in a solid media in the presence of a first substrate, one or more other enzymes and a first dye, and wherein a product of the chemical reaction between the enzyme of interest and the first substrate is a substrate for one of the other enzymes, and wherein the first dye is

a substrate for one of the other enzymes, and wherein the product of the chemical reaction between the first dye and one of the other enzyme is a second dye, and wherein the colour of the first dye is different from the colour of the second dye.

Furthermore, the present invention also provides for a method for testing or screening for an activity of an enzyme of interest comprising testing of screening for a change in a colour of a first dye by the presence of a host cell expressing an enzyme of interest, wherein the host cell has been cultivated on a solid media in the presence of a first dye and a polymer, wherein the product of the chemical reaction between the first dye and the enzyme of interest is a second dye, and wherein the polymer is capable of binding to the second dye, and wherein the colour of the first dye is different from the colour of the second dye.

DEFINITIONS

In the context of the present invention, the term "E.C." (Enzyme Class) refers to the internationally recognized enzyme classification system, Recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, Academic Press, Inc. Unless otherwise indicated, it refers to the version of this recommendation from 1992. For those enzymes which are not present in the 1992-version it refers to the version of said recommendation found as the web-edition in January 2004, however for these enzymes the reaction that the enzyme catalyzes is also indicated.

The term "substrate" or "substrate for an enzyme" is in the context of the present invention to be understood as a compound for which a chemical reaction converting the substrate into a product is catalysed by an enzyme.

The term "product" is in the context of the present invention to be understood as a compound produced by the chemical reaction converting a substrate into a product catalysed by an enzyme. The "substrate" and "product" of a chemical reaction catalysed by an enzyme are two chemically different compounds.

The term "nucleic acid sequence" is in the context of the present invention to be understood as a single-or double-stranded deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) polynucleotide.

In the context of the present invention the term "a host cell expressing an enzyme" or "expression of an enzyme by a host cell" is to be understood as transcription and translation of a nucleic acid sequence encoding the enzyme of interest. Depending on the particular enzyme it may also include e.g. extracellular secretion of the enzyme by the host cells secretory pathway or transport to a intracellular organelle of the host cell. The enzyme may be e.g. the enzyme of interest.

The term "colour zone" used in relation to the host cells refers in the context of the present invention to a zone or area surrounding the host cell where a change in the colour of the solid media may be seen as a result of expression of the enzyme of interest.

The term "binding" or "bound" is in the context of the present invention to be understood as the establishment or the presence of any type of chemical and/or physical bond between two molecules, such as a covalent bond, a hydrogen bond, an electrostatic bond or ionic bond, or the attraction of molecules to each other by van der Waals forces.

DETAILED DESCRIPTION OF THE INVENTION

Method of the invention

One embodiment of the present invention relates to a method for testing or screening a host cell for expression of an enzyme of interest, comprising testing or screening for a change in the colour of a first dye by the presence of a host cell expressing the enzyme of interest, wherein the host cell has been cultivated on or in a solid media in the presence of a first substrate, one or more other enzymes and a first dye, and wherein a product of the chemical reaction between the enzyme of interest and the first substrate is a substrate for one of the other enzymes, and wherein the first dye is a substrate for one of the other enzymes, and wherein the product of the chemical reaction between the first dye and one of the other enzyme is a second dye, and wherein the colour of the first dye is different from the colour of the second dye.

Thus schematically shown the following chemical reactions take place:

first substrate + enzyme of interest -> (product + other enzyme)$_n$ -> product + other enzyme + first dye -> second dye,

wherein "n" indicates the number of chemical reactions between a product and an other enzyme, wherein the product of each chemical reaction is a substrate for another other enzyme. Typically "n" may be 0, 1, 2, 3 or 4. More particularly "n" may be 0, 1 or 2. Above equation is only meant as an illustration showing the most relevant components of each chemical reaction, i.e. this means that e.g. the enzymes involved are only shown as reactants and/or some of the products of a chemical reaction may not be shown.

An advantage of said method is that it is not necessary to have a substrate for the enzyme of interest which is capable of changing colour upon reaction with the enzyme of interest. Furthermore, it is not necessary with the present method to use a substrate labelled with a dye.

In particular the chemical reaction between the other enzyme and the first dye may be a redox reaction; more particularly the other enzyme may catalyze oxidation of the first dye into a second dye.

In a particular embodiment said method may be performed in the presence of a polymer capable of binding the second dye, e.g. a polymer capable of binding the second dye may be present in the solid media. As described below the inventor of the present invention has found that the presence of said polymer may fixate the formed second dye in the solid media so that it does not diffuse. Diffusion of the formed second dye is a well-known problem for many methods related to testing or screening for an enzymatic activity.
Above method may comprise the following steps:

a) cultivating a host cell expressing the enzyme of interest on or in a solid media in the presence of a substrate for the enzyme, one or more other enzymes and a dye, wherein the product of the reaction between the substrate and the enzyme is a substrate for one of the other enzymes, and wherein the dye is a substrate for one of the other enzymes, and wherein the product of the reaction between one of the other enzymes and the dye has a different colour than the compound.

b) identifying a host cell by the presence of the colour of the second dye

Another embodiment of the present invention relates to a method for testing or screening for an activity of an enzyme of interest comprising testing of screening for a change in a colour of a first dye by the presence of a host cell expressing an enzyme of interest, wherein the host cell has been cultivated on or in a solid media in the presence of a first dye and a polymer, wherein the product of the chemical reaction between the first dye and the enzyme of interest is a second dye, and wherein the polymer is capable of binding to the second dye, and wherein the colour of the first dye is different from the colour of the second dye.
Said method may comprise the following steps:

a) cultivating a host cell expressing the enzyme on or in a solid media in the presence of a first dye and a polymer, wherein the product of the chemical reaction between the first dye and the enzyme of interest is a second dye, and wherein the polymer is capable of binding to the second dye, and wherein the colour of the first dye is different from the colour of the second dye.

b) identifying a host cell by the presence of the colour of the second dye

The inventor of the present invention have found that the presence of a polymer capable of binding the second dye can reduce the diffusion of the second dye in the solid media and thereby ease detection of host cells expressing the enzyme of interest. Thus the use of a

polymer may aid in distinguishing host cells expressing the enzyme of interest from host cells not expressing said enzyme. The presence of the polymer is particularly advantageous if the colour zone surrounding the host cell does not have a well-defined border.

In yet another embodiment the present invention relates to a method for testing or screening for an activity of a peroxidase (E.C. 1.11.1.7) comprising testing of screening for a change in the colour of Brilliant Blue by the presence of a host cell expressing the peroxidase, wherein the host cell has been cultivated on or in a solid media in the presence of Brilliant Blue and hydrogen peroxide.

The inventor of the present invention has found that Brilliant Blue is particularly useful to detect peroxidase activity from host cells cultured on or in a solid media.

The methods of the present invention may be used to test the enzymatic activity of one enzyme or it may be used to screen for an enzymatic activity. In particular the methods of the present invention may be used to screen a library of host cells for expression of a particular enzymatic activity. For example said methods may be used to screen for mutants of known enzymes or to screen one or more organism for expression of a particular enzymatic activity. This may performed by creation of a library of nucleic acid sequences encoding different mutants of a known enzyme or a library of nucleic acid sequences encoding different polypeptides expressed by a particular organism and subsequently introducing said library into a host cell.

Enzyme of interest

In one embodiment of the present invention the enzyme of interest may be any enzyme for which a product of the chemical reaction between the enzyme of interest and a substrate (i.e. a first substrate) is a substrate for another enzyme.

For example the enzyme of interest may in particular be glucosidase(E.C. 3.2.1), such as a glucan 1,4-alpha-glucosidase (also known as glucoamylase) (E.C. 3.2.1.3), e.g. the G1 or G2 form of the glycoamylase from *Aspergillus niger* (SWISSPROT:AMYG_ASPNG Prim. accession # P04064 and reference "Glucoamylases G1 and G2 from *Aspergillus niger* are synthesized from two different but closely related mRNAs", Boel,E et al. (1984), EMBO J. 3:1097), or it may be the glycoamylase from *Talaromyces emersonii* (WO9928448). It may also be an alpha-amylase (E.C. 3.2.1.1), such as a *Bacillus* alpha-amylase (often referred to as "Termamyl-like alpha-amylases"). Well-known Termamyl-like alpha-amylases include alpha-amylase derived from a strain of *B. licheniformis* (commercially available as Termamyl), *B. amyloliquefaciens,* and *B. stearothermophilus* alpha-amylase. Other Termamyl-like alpha-amylases include alpha-amylase derived from a strain of the *Bacillus* sp. NCIB 12289, NCIB 12512, NCIB 12513 or DSM 9375, all of which are described in detail in WO95/26397, and the alpha-amylase described by Tsukamoto et al., Biochemical and Biophysical Research Communications, 151 (1988), pp. 25-31. In the context of the present invention a Termamyl-like alpha-

amylase is an alpha-amylase as defined in WO99/19467 on page 3, line 18 to page 6, line 27. Contemplated variants and hybrids are described in WO96/23874, WO97/41213, and WO99/19467. Specifically contemplated is a recombinant *B.stearothermophilus* alpha-amylase variant with the mutations: I181* + G182* + N193F. *Bacillus* alpha-amylases may be added in effective amounts well known to the person skilled in the art.

It may also be a beta-amylase (E.C. 3.2.1.2), such as a beta-amylase obtained from wheat or barley, e.g. Novozym WBA®, or a glucan 1,4-alpha-maltohydrolase (E.C. 3.2.1.133), i.e. an exo-acting maltogenic alpha-amylase as described in WO 9104669 or WO 9943794 of which an example is Novamyl® (product of Novozymes A/S).

The enzyme of interest may also be a pectinesterase (E.C. 3.1.1.11), a cellulase (E.C. 3.2.1.4), a beta-glucosidase (E.C. 3.2.1.21), an alpha-galactosidase (E.C. 3.2.1.22), a glucan 1,3-beta-glucosidase (E.C. 3.2.1.58), a cellulose 1,4-beta-cellobiosidase (E.C. 3.2.1.91), a lactase (E.C. 3.2.1.108), a beta-galactofuranosidase (the web-edition from January 2004: E.C. 3.2.1.146; enzymes catalyzing the reaction of: Hydrolysis of terminal non-reducing b-D-galactofuranosides, releasing galactose), a carboxypeptidase A (E.C. 3.4.17.1) or a aldose 1-epimerase (E.C. 5.1.3.3).

In another particular embodiment the enzyme of interest may be an enzyme capable of producing hydrogen peroxide upon reaction with a substrate for the enzyme. In particular it may be an oxidase, such as a malate oxidase (E.C. 1.1.3.3), a glucose oxidase (E.C. 1.1.3.4), e.g. glucose oxidase derived from *Aspergillus niger*, hexose oxidase (E.C. 1.1.3.5), cholesterol oxidase (E.C. 1.1.3.6), aryl-alcohol oxidase (E.C. 1.1.3.7), L-gulonolactone oxidase (E.C. 1.1.3.8), galactose oxidase (E.C. 1.1.3.9), pyranose oxidase (E.C. 1.1.3.10), L-sorbose oxidase (E.C. 1.1.3.11), pyridoxine oxidase (E.C. 1.1.3.12), alcohol oxidase (E.C. 1.1.3.13), (S)-2-hydroxy-acid oxidase (E.C. 1.1.3.15), ecdysone oxidase (E.C. 1.1.3.16), choline oxidase (E.C. 1.1.3.17), secondary-alcohol oxidase (E.C. 1.1.3.18), 4-hydroxymandalate oxidase (E.C. 1.1.3.19), glycerol-3-phosphate oxidase (E.C. 1.1.3.21), xanthine oxidase (E.C. 1.1.3.22), thiamine oxidase (E.C. 1.1.3.23), L-galactonolactone oxidase (E.C. 1.1.3.24), cellobiose oxidase (E.C. 1.1.3.25), hydroxypythanate oxidase (E.C. 1.1.3.27), N-acylhexosamine oxidase (E.C. 1.1.3.29), polyvinyl-alcohol oxidase (E.C. 1.1.3.30), D-arabino-1,4-lactone oxidase (the web-edition from January 2004: E.C. 1.1.3.37; enzymes catalyzing the reaction of: D-arabinono-1,4-lactone + O2 -> D-*erythro*-ascorbate + H2O2), vanillyl-alcohol oxidase (the web-edition from January 2004: E.C. 1.1.3.38; enzymes catalyzing the reaction of: vanillyl alcohol + O2 -> vanillin + H2O2), nucleoside oxidase (H2O2 – forming) (the web-edition from January 2004: E.C. 1.1.3.39; enzymes catalyzing the reaction of: adenosine + 2 O2 -> 9-riburonosyladenine + 2 H2O2), D-mannitol oxidase (the web-edition from January 2004: E.C. 1.1.3.40; enzymes catalyzing the reaction of: mannitol + O2 -> mannose + H2O2) or xylitol

oxidase (the web-edition from January 2004: E.C. 1.1.3.41; enzymes catalyzing the reaction of: xylitol + O2 -> xylose + H2O2).

Other examples of relevant oxidases include, but are not limited to, aldehyde oxidase (E.C. 1.2.3.1), Pyruvate oxidase (E.C.1.2.3.3), Oxalate oxidase (E.C. 1.2.3.4), Glyoxylate oxidase (E.C. 1.2.3.5), Pyruvate oxidase (CoA-acetylating) (E.C. 1.2.3.6), Aryl-aldehyde oxidase (E.C. 1.2.3.9), Retinal oxidase (E.C. 1.2.3.11), Dihydroorotate oxidase. (E.C. 1.3.3.1), Lathosterol oxidase (E.C.1.3.3.2), Acyl-CoA oxidase (E.C. 1.3.3.6), Dihydrouracil oxidase (E.C.1.3.3.7), Tetrahydroberberine oxidase (E.C.1.3.3.8), D-aspartate oxidase (E.C.1.4.3.1), L-amino acid oxidase (E.C.1.4.3.2), D-amino acid oxidase (E.C.1.4.3.3), Amine oxidase (flavin-containing) (E.C. 1.4.3.4), Pyridoxamine-phosphate oxidase (E.C. 1.4.3.5), Amine oxidase (copper-containing) (E.C.1.4.3.6), D-glutamate oxidase (E.C.1.4.3.7), Ethanolamine oxidase (E.C.1.4.3.8), Putrescine oxidase (E.C. 1.4.3.10), L-glutamate oxidase (E.C.1.4.3.11), Cyclohexylamine oxidase (E.C.1.4.3.12), Protein-lysine 6-oxidase. (E.C.1.4.3.13), L-lysine oxidase (E.C.1.4.3.14), D-glutamate(D-aspartate) oxidase (E.C.1.4.3.15), L-aspartate oxidase (E.C.1.4.3.16), Glycine oxidase (the web-edition from January 2004: E.C.1.4.3.19; enzymes capable of catalyzing one of the following reactions: (1) glycine + H2O + O2 -> glyoxylate + NH3 + H2O2; (2) D-alanine + H2O + O2 -> pyruvate + NH3 + H2O2; (3) sarcosine + H2O + O2 -> glyoxylate + methylamine + H2O2; (4) *N*-ethylglycine + H2O + O2 -> glyoxylate + ethylamine + H2O2), Sarcosine oxidase (E.C. 1.5.3.1), N-methyl-L-amino-acid oxidase (E.C.1.5.3.2), N(6)-methyl-lysine oxidase (E.C.1.5.3.4), (S)-6-hydroxynicotine oxidase (E.C.1.5.3.5), (R)-6-hydroxynicotine oxidase (E.C.1.5.3.6), L-pipecolate oxidase (E.C.1.5.3.7), Dimethylglycine oxidase (E.C.1.5.3.10), Polyamine oxidase (E.C.1.5.3.11), Dihydrobenzophenanthridine oxidase (the web-edition from January 2004: E.C.1.5.3.12; enzymes capable of catalyzing one of the following reactions: (1) dihydrosanguinarine + O2 -> sanguinarine + H2O2; (2) dihydrochelirubine + O2 -> chelirubine + H2O2; (3) dihydromacarpine + O2 -> macarpine + H2O2), NADPH oxidase (the web-edition from January 2004: E.C.1.6.3.1; enzymes capable of catalyzing the reaction of: $NAD(P)H^+ H^+ + O2 \rightarrow NAD(P)^+ + H2O2$).

In another embodiment of the present invention the enzyme of interest may be any enzyme for which a product of the chemical reaction between the enzyme of interest and a first dye is a second dye, wherein the colour of the first dye is different from the colour of the second dye. In particular the enzyme of interest for this method may be a peroxidase (E.C. 1.11.1.7). Peroxidase is an enzyme capable of catalyzing the oxidation, by hydrogen peroxide, of a number of substrates such as ascorbate, cytochrome C and the leuco form of many dyes. A representative reaction is shown below:

Peroxidase + H2O2 + DH2 ------> 2 H2O + D,

wherein DH2 = leuco dye and D = dye

In particular the peroxidase may be Horseradish peroxidase (HRP) which exists in the form of several isozymes, all containing heme as the prosthetic group. The enzyme has a molecular weight of approximately 40,000. For example it may be the Horseradish peroxidase from Sigma-Aldrich, product number P8125.

The ability of peroxidase to catalyze the oxidation of a number of organic compounds by hydrogen peroxide, resulting in formation of colored end-products, is utilized in several methods of determination of glucose and galactose in biological fluids.

For both methods of the present invention the enzyme of interest may be naturally expressed by a host cell or it may be an enzyme which a host cell has been manipulated to express, i.e. by introduction of a nucleic acid sequence encoding the enzyme of interest into the host cells. Thus the nucleic acid sequence encoding the enzyme of interest, i.e. the nucleic acid sequence of interest may be native or foreign to the host cell. In this context the term "native" refers to a nucleic acid sequence which is naturally present in the genome of the host cell, while the term "foreign" refers to a nucleic acid sequence which is not normally present in the host cell of the invention, i.e. a nucleic acid sequence which has been introduced into the genome of the host cell.

In a particular embodiment the host cells of the present invention may be express a library of nucleic acid sequences. For example said library may represent mutations of a particular nucleic acid sequence or they may represent different nucleic acid sequences obtained from one or more different organisms. Methods for introducing a library of nucleic acid sequences into a host cell are well-known for a person skilled in the art.

Methods for introducing a nucleic acid sequence into a host cell typically includes cloning the nucleic acid sequence encoding the enzyme of interest into an expression vector, introducing the expression vector into a host cell and culturing the host cells under conditions suitable for expression of the enzyme of interest. Methods for cloning of nucleic acid sequences, introducing a nucleic acid sequence or expression vector into a host and culturing the host cell under conditions suitable for expression of the enzyme of interest generally depends on the enzyme of interest, the expression vector and/or the host cell. Such methods are well known to a person skilled in the art and may for example be found in "Molecular cloning: A laboratory manual", Sambrook et al. (1989), Cold Spring Harbor lab., Cold Spring Harbor, NY; Ausubel, F. M. et al. (eds.); "Current protocols in Molecular Biology", John Wiley and Sons, (1995); Harwood, C. R., and Cutting, S. M. (eds.); "Molecular Biological Methods for Bacillus", John Wiley and Sons, (1990); "DNA Cloning: A Practical Approach, Volumes I and II", D.N. Glover ed. (1985); "Oligonucleotide Synthesis", M.J. Gait ed. (1984); "Nucleic Acid Hybridization", B.D. Hames & S.J. Higgins eds (1985); "Transcription And Translation", B.D. Hames & S.J. Higgins, eds.

(1984); "Animal Cell Culture", R.I. Freshney, ed. (1986); "Immobilized Cells And Enzymes", IRL Press, (1986); "A Practical Guide To Molecular Cloning", B. Perbal, (1984).

As described previously the nucleic acid sequence of interest is a single-or double-stranded DNA or RNA polynucleotide. The enzyme of interest may in particular be encoded by a genomic DNA or a cDNA sequence.

The enzyme of interest may derive from any cell, including but not limited to one of those described as host cells below.

First substrate

The first substrate of the present invention may be any compound which is a substrate for the enzyme of interest. Thus the choice of the first substrate depends on the enzyme of interest. Substrates for different enzymes are known to a person skilled in the art.

In one embodiment of the present invention the product of the reaction between the enzyme of interest and the first substrate should be a substrate for one of the other enzyme(s).

For example if the enzyme of interest in this embodiment is a glucoamylase (E.C. 3.2.1.3) the first substrate may for example be selected from the group consisting of but not limited to: maltose, maltodextrin, starch, e.g. potato starch.

If the enzyme of interest is an alpha-amylase (E.C. 3.2.1.1) or a beta-amylase (E.C.3.2.1.2) or a glucan 1,4-alpha-maltohydrolase (E.C. 3.2.1.133) the first substrate may be maltotriose.

If the enzyme of interest is a cellulase (E.C. 3.2.1.4) the first substrate may be cellulose.

If the enzyme of interest is a glucose oxidase (E.C. 1.1.3.4.) the first substrate may be beta-D-glucose.

If the enzyme of interest is a galactose oxidase (E.C. 1.1.3.9) the first substrate may be D-galactose.

If the enzyme of interest is an alcohol oxidase (E.C. 1.1.3.13) the first substrate may be a primary alcohol.

If the enzyme of interest is a L-amino acid oxidase (E.C. 1.4.3.2) the first substrate may be an L-amino acid.

In another embodiment of the present invention the enzyme of interest may be any enzyme for which a product of the chemical reaction between the enzyme of interest and a first dye is a second dye, wherein the colour of the first dye is different from the colour of the second dye. Thus if the enzyme of interest is peroxidase (E.C. 1.11.1.7) the first substrate may be a first dye, in particular it may be a compound which is capable of being oxidized by peroxidase into a second dye. Examples of such first dyes are given below.

Other enzymes

One embodiment of the present invention relates to a method for detection of the activity of an enzyme comprising using a first substrate, one or more other enzymes and a first dye. The principle behind this method is that a product produced by the conversion of the first substrate to a product catalysed by the enzyme of interest is a substrate for one of the other enzymes. The product produced by the conversion of this substrate catalysed by one of the other enzymes may then again be a substrate for one of the other enzymes. In this way a chain of chemical reactions converting a substrate to a product catalysed by an enzyme is created. Thus in principle the method utilizes a cascade of chemical reactions catalyzed by enzymes leading from the first chemical reaction between the enzyme of interest and the first substrate to the last chemical reaction between one of the other enzymes and the first dye. As previously shown this may be shown schematically in the following way:

first substrate + enzyme of interest -> (product + other enzyme)$_n$ -> product + other enzyme + first dye -> second dye,

wherein "n" indicates the number of chemical reactions between a product and an other enzyme, wherein the product of each chemical reaction is a substrate for another other enzyme. Typically "n" may be 0, 1, 2, 3 or 4. More particularly "n" may be 0, 1 or 2.

The choice of the other enzymes depends on the particular enzyme of interest and the first substrate. However, in a particular embodiment the other enzymes may comprise at least a peroxidase.

In another particular embodiment the other enzymes may comprise at least an enzyme capable of producing hydrogen peroxide upon reaction with a substrate for the enzyme, and a peroxidase. In this case the first dye may particularly be a compound capable of being oxidized to a second dye. Examples of peroxidase of particular interest are those described above as enzyme of interest.

For example, for testing or screening for a glucoamylase activity the enzyme of interest may be a glucoamylase (E.C. 3.2.1..3), the first substrate may be maltose, maltodextrin or a starch, e.g. potato starch, the other enzymes may be a glucose oxidase (E.C. 1.1.3.4) and a peroxidase (E.C. 1.11.1.7) and the first dye may be a compound capable of being oxidized by a peroxidase, e.g. Brilliant Blue. Thus in this case the following cascade of enzyme catalysed chemical reactions may take place:

Maltose + glucoamylase -> Glucose + Glucose Oxidase -> H2O2 + first dye (red) + Peroxidase -> second dye (ox)

wherein the term "(red)" and "(ox)" refers to the reduced and oxidized forms of the same compound.

Or for testing or screening for a cellulase activity the enzyme of interest may be a cellulase (E.C.3.2.1.4), the first substrate may be cellulose, the other enzymes may be cellobiose oxidase (E.C. 1.1.3.25) and a peroxidase (E.C. 1.11.1.7), and the first dye may be a compound capable of being oxidized by a peroxidase. Thus in this case the following cascade of enzyme catalysed chemical reactions may take place:

Cellulose + cellulase -> cellobiose + cellobiose oxidase -> H2O2 + first dye (red) + Peroxidase -> second dye (ox)

wherein the term "(red)" and "(ox)" refers to the reduced and oxidized forms of the same compound.

Another way of testing or screening for a cellulase activity include using the same components as described above but with the exception of using a beta-glucosidase (E.C. 3.2.1.21) and a glucose oxidase (E.C. 1.1.3.4) instead of a cellobiose oxidase as a other enzyme(s). Thus in this case the following reaction takes place:

Cellulose + cellulase -> cellobiose + beta-glucosidase -> glucose + glucose oxidase ->H2O2 + first dye (red) + Peroxidase -> second dye (ox)

wherein the term "(red)" and "(ox)" refers to the reduced and oxidized forms of the same compound.

Similarly, for testing or screening for a beta-glucosidase activity the above reaction may be used with a beta-glucosidase as enzyme of interest (E.C. 3.2.1.21), cellobiose as the first substrate, a glucose oxidase (E.C. 1.1.3.4) and a peroxidase (E.C. 1.11.1.7) as the other enzymes and a compound capable of being oxidized by a peroxidase as the first dye. Thus in this case the following cascade of enzyme catalysed chemical reactions may take place:

Cellobiose + beta-glucosidase -> glucose + glucose oxidase ->H2O2 + first dye (red) + Peroxidase -> second dye (ox)

wherein the term "(red)" and "(ox)" refers to the reduced and oxidized forms of the same compound.

If the enzyme of interest is a pectinesterase (E.C. 3.1.1.11), the first substrate may e.g. be pectin, the other enzymes may be an alcohol oxidase (E.C. 1.1.3.13) and a peroxidase (E.C.

1.11.1.7), and the first dye may be a compound capable of being oxidized by a peroxidase. Thus, in this case the following cascade of enzyme catalysed chemical reactions may take place:

Pectin + pectinesterase -> methanol + alcohol oxidase -> H2O2 + first dye (red) + Peroxidase -> second dye (ox),

wherein the term "(red)" and "(ox)" refers to the reduced and oxidized forms of the same compound.

Or if the enzyme of interest is an alpha-galactosidase (E.C. 3.2.1.22), the first substrate may be melibiose, the other enzymes may be a glucose oxidase (E.C. 1.1.3.4) and a peroxidase (E.C. 1.11.1.7) or the other enzymes may be a galactose oxidase (E.C. 1.1.3.9) and a peroxidase (E.C. 1.11.1.7), and the first dye may be a compound capable of being oxidized by a peroxidase. Thus in this case one of the following cascades of enzyme catalysed chemical reactions may take place:

Melibiose + alpha-galactosidase -> glucose + galactose + glucose oxidase -> H2O2 + first dye (red) + Peroxidase -> second dye (ox),

or

Melibiose + alpha-galactosidase -> glucose + galactose + galatose oxidase -> H2O2 + first dye (red) + Peroxidase -> second dye (ox),

wherein the term "(red)" and "(ox)" refers to the reduced and oxidized forms of the same compound.

If the enzyme of interest is a cellulose 1,4-beta-cellobiosidase (E.C. 3.2.1.91), the first substrate may be cellulose or cellotetraose, the other enzymes may be a cellobiose oxidase (E.C. 1.1.3.25) and a peroxidase (E.C. 1.11.1.7), and the first dye may be a compound capable of being oxidized by a peroxidase. Thus with cellulose as the first substrate the following cascade of enzyme catalysed chemical reactions may take place:

Cellulose + cellulose 1,4-beta-cellobiosidase -> Cellobiose + cellobiose oxidase -> H2O2 + first dye (red) + Peroxidase -> second dye (ox)

wherein the term "(red)" and "(ox)" refers to the reduced and oxidized forms of the same compound.

Another way for testing or screening for the activity of a cellulose 1,4-beta-cellobiosidase (E.C. 3.2.1.91) include using the same components as described above but with the exception that a beta-glucosidase (E.C. 3.2.1.21), a glucose oxidase (E.C. 1.1.3.4) and a peroxidase (E.C. 1.11.1.7) are used as the other enzyme instead of the cellobiose oxidase and the peroxidase. Thus with cellulose as the first substrate the following cascade of enzyme catalysed chemical reactions may take place:

Cellulose + cellulose 1,4-beta-cellobiosidase -> Cellobiose + beta-glucosidase -> glucose + glucose oxidase -> H2O2 + first dye (red) + Peroxidase -> second dye (ox)

wherein the term "(red)" and "(ox)" refers to the reduced and oxidized forms of the same compound.

If the enzyme of interest is a lactase (E.C. 3.2.1.108), the first substrate may be lactose, the other enzymes may be a glucose oxidase (E.C. 1.1.3.4) and a peroxidase (E.C. 1.11.1.7) or the other enzymes may be a galactose oxidase (E.C. 1.1.3.9) and a peroxidase (E.C. 1.11.1.7), and the first dye may be a compound capable of being oxidized by a peroxidase. Thus in this case one of the following cascades of enzyme catalysed chemical reactions may take place:

Lactose + lactase -> glucose + galactose + glucose oxidase -> H2O2 + first dye (red) + Peroxidase -> second dye (ox),

or

Lactose + lactase -> glucose + galactose + galatose oxidase -> H2O2 + first dye (red) + Peroxidase -> second dye (ox),

wherein the term "(red)" and "(ox)" refers to the reduced and oxidized forms of the same compound.

If the enzyme of interest is a beta-galactofuranosidase (the web-edition from January 2004: E.C. 3.2.1.146), the first substrate may be beta-D-galactofuranoside, the other enzymes may be a galactose oxidase (E.C. 1.1.3.9) and a peroxidase (E.C. 1.11.1.7), and the first dye may be a compound capable of being oxidized by a peroxidase. Thus in this case one of the following cascades of enzyme catalysed chemical reactions may take place:

beta-D-galactofuranoside + beta-galactofuranosidase -> galactose + galactose oxidase -> H2O2 + first dye (red) + Peroxidase -> second dye (ox),

wherein the term "(red)" and "(ox)" refers to the reduced and oxidized forms of the same compound.

If the enzyme of interest is a carboxypeptidase A (E.C. 3.4.17.1), the first substrate may be a polypeptide, the other enzymes may be an L-amino acid oxidase (E.C. 1.4.3.2) and a peroxidase (E.C. 1.11.1.7), and the first dye may be a compound capable of being oxidized by a peroxidase. Thus in this case the following cascade of enzyme catalysed chemical reactions may take place:

Polypeptide + carboxypeptidase A -> amino acid + L-amino acid oxidase -> H2O2 + first dye (red) + Peroxidase -> second dye (ox),

wherein the term "(red)" and "(ox)" refers to the reduced and oxidized forms of the same compound.

For testing or screening for the activity of an oxidase the enzyme of interest may be an oxidase, e.g. one of those described above, the first substrate may be any compound which is a substrate for the particular oxidase of choice, the other enzyme may be a peroxidase (E.C. 1.11.1.7) and the first dye may be a compound capable of being oxidized by the peroxidase. For example if the oxidase is a glucose oxidase and the first substrate is beta-D-glucose the following cascade of enzyme catalysed chemical reactions may take place:

Glucose + Glucose Oxidase -> H2O2 + first dye (red) + Peroxidase-> second dye (ox),

wherein the term "(red)" and "(ox)" refers to the reduced and oxidized forms of the same compound. Similar reactions may take place when the enzyme of interest is another oxidase and the first substrate is a substrate for the oxidase.

Dye

The first dye of the present invention should be a substrate for an enzyme for which a second dye is a product of the chemical reaction between the enzyme and the first dye and wherein the second dye has a different colour than the first dye. Said enzyme may be another enzyme or an enzyme of interest, depending on for which method of the present invention the first dye is used for.

Schematically shown the first dye should be capable of being involved in the following chemical reaction:

First dye + enzyme -> second dye

The term "colour" refers in the context of the present invention either to colours which are visible to the human eye (i.e. electromagnetic radiation with a wavelength of between 400 and 700 nm) or to the emission of a fluorescent signal by the first and/or second dye.

If white light is spread out by a prism, we can see that it is composed of different colours. Each colour corresponds to a different wavelength. The colour of a compound depends on the wavelength of light which it absorbs. If a compound does not absorb any visible light it will be colourless. If a compound absorbs light humans perceive the complementary colour, because the light which reaches our eyes is missing the wavelengths which have been absorbed.

The relation between some wavelengths of visible light and colour are:

Wavelength of light, nm	Colour	Complementary colour
400-430	Violet	Green-yellow
430-480	Blue	Yellow
480-490	Green-blue	Orange
490-510	Blue-green	Red
510-530	Green	Purple
530-570	Yellow-green	Violet
570-580	Yellow	Blue
580-600	Orange	Green-blue
600-680	Red	Blue-green

Thus the term "different colour" refers in this context to that the first and second dye absorbs different wavelengths of visible light or that the first dye does not absorb any light within the visible area (i.e. it is colour-less), while the second dye does absorb light of the visible area or vice versa.

The first and/or second dye may also be a compound capable of emitting a fluorescent signal. Emission of fluorescence is achieved by excitation of the fluorescent compound using a specific electromagnetic radiation wavelength, the specific wavelength depends on the compound. The emission wavelength is different from the excitation wavelength. In this context the term "different colour" in reference to the first and second dye refers then to the ability of the first dye to emit fluorescent light, while the second dye does not or vice versa, or that the intensity of the fluorescent light emitted from the first dye is either increased or decreased as compared to the intensity of the fluorescent light emitted from the second dye.

In a particular embodiment of the present invention the enzyme which reacts with the first dye is a peroxidase, i.e. the first dye is a compound capable of being oxidized by a peroxidase (E.C. 1.11.1.7) to a second dye. Thus the first dye is a reduced form of a compound and the second dye is the oxidized form of the compound.

Examples of first dyes which are capable of being oxidized to a second dye in the presence of a peroxidase (and hydrogen peroxide) include, but are not limited to: Dyes of the class Triphenyl methane; e.g. Crystalviolet, Malachite green or bromophenol blue, or dyes of Azo class; e.g. Methyl orange or Congo red, or Phenol based dyes; Phenol red, or Polymeric dyes; e.g. Poly R478; Anthraquinone-based; e.g. Remazol Brilliant Blue, or cationic dyes; e.g. ruthenium red, or anionic dyes; e.g. eosin.

Above examples of first dyes are all dyes where the change in colour from the first to the second dye is change which is detectable by visible light. However, as described above the change in colour from the first to the second dye may also relate to a fluorescent signal. In this case if the first dye should be a substrate for a peroxidase the first dye may be a non-oxidized form of a compound which upon oxidation by a peroxidase emits a fluorescent signal with an intensity which is either increased or decreased as compared to the emission wavelength of the non-oxidised form (first dye). The difference in the emission of the oxidised form (second dye) may then be detected in a background of the non-oxidized form (first dye). Examples of first dyes which comprise this ability include but are not limited to 10-Acetyl-3,7-dihydroxyphenoxazine (Amplex red TM from Molecular Probes, USA) In the presence of horseradish peroxidase (HRP), the Amplex Red reagent reacts in a 1:1 stoichiometry with H_2O_2 to produce highly fluorescent resorufin (Optimal Excitation wavelength is nm544 and optimal emission wavelength is nm590).

Polymer

The polymer of the present invention may be any polymer capable of binding the second dye. An advantage of using a polymer capable of binding the second dye is that diffusion of the second dye in the solid media is reduced, thereby making it easier to detect host cells expressing an enzyme of interest. This may be of particular importance if the border of the colour-zone surrounding the host cells is difficult to define.

Examples of suitable polymers include but are not limited to: carboxymethyl-cellulose (CMC), chitin, pectate, pectin, starch e.g. potato starch, locust bean gum and ghatti gum.

Typically the choice of polymer depends on the identity of the second dye. Thus for example if the first dye is Brilliant Blue or Congo Red the polymer may in particular be CMC, chitin, pectate, pectin or starch.

If the first dye is Ruthenium Red the polymer may in particular be pectate or pectin.

If the first dye is Eosin the polymer may in particular be chitin or chitosan.

Host cells

The enzyme of interest may be expressed by any host cell. The host cell may a prokaryotic or eukaryotic cell, for example it may be a bacterium, fungus, such as yeast or a filamentous fun-

gus, mammalian, plant or an insect cell. The enzyme may be native or foreign to the host cell, i.e. it may be expressed naturally by the host cell or it may be an enzyme the host cells does not express naturally. The host cell may have been manipulated to express the enzyme of interest, e.g. by introducing a nucleic acid sequence encoding the enzyme of interest into the host cell, or the host cell may be cell which naturally expresses the enzyme of interest.

Examples of bacterial host cells include gram-positive bacteria such as a strain of *Bacillus*, e.g. strains of *B. subtilis*, *B. licheniformis*, *B. lentus*, *B. brevis*, *B. stearothermophilus*, *B. alkalophilus*, *B. amyloliquefaciens*, *B. coagulans*, *B. circulans*, *B. lautus*, *B. megaterium* or *B. thuringiensis*, or strains of *Streptomyces*, such as *S. lividans* or *S. murinus*, or gram-negative bacteria such as *Escherichia coli* or *Pseudomonas sp.*

Transformation of bacteria may be effected by protoplast transformation, electroporation, conjugation, or by using competent cells in a manner known per se (cf. Sambrook et al., supra).

If the enzyme of interest is expressed by gram-positive bacteria such as a *Bacillus* or *Streptomyces* strain, the enzyme may be retained in the cytoplasm, or it may be directed to the extracellular medium by a bacterial secretion sequence.

If the enzyme of interest is expressed by a gram-negative bacteria such as *E. coli*, the enzyme may be retained in the cytoplasm, typically as insoluble granules (known as inclusion bodies), or it may be directed to the periplasmic space by a bacterial secretion sequence. In the former case, the cells may typically be lysed. In the latter case, the enzyme may be released from the periplasmic space by disrupting the cells, e.g. by sonication or osmotic shock, to release the contents of the periplasmic space.

Examples of host yeast cells include cells of a species of *Candida*, e.g. *C. maltose*, or *Kluyveromyces*, e.g. *K. lactis*, *K. fragilis*, or *Saccharomyces*, e.g. *S. carlsbergensis*, *S. cerevisiae*, *S. diastaticus*, *S. douglasii*, *S. kluyveri*, *S. norbensis* or *S. oviformis*, or *Schizosaccharomyces*, e.g. *S. pombe*, or *Pichia*, e.g. *P. pastoris*, *P. guillermondii* or *P. methanolio*, or *Hansenula*, e.g. *H. polymorpha*, or *Yarrowia*, e.g. *Y. lipolytica* or *Ustilgo maylis* (cf. Gleeson et al., J. Gen. Microbiol. 132, 1986, pp. 3459-3465; US 4,882,279 and US 4,879,231). Since the classification of yeast may change in the future, for the purposes of this invention, yeast shall be defined as described in Biology and Activities of Yeast (Skinner, F.A., Passmore, S.M., and Davenport, R.R., eds, Soc. App. Bacte-riol, Symposium Series No. 9, 1980. The biology of yeast and manipulation of yeast genetics are well known in the art (see, e.g., Biochemistry and Genetics of Yeast, Bacil, M., Horecker, B.J., and Stopani, A.O.M., editors, 2nd edition, 1987; The Yeasts, Rose, A.H., and Harrison, J.S., editors, 2nd edition, 1987; and The Molecular Biology of the Yeast Saccharomyces, Strathern et al., editors, 1981). Yeast may be transformed using the procedures described by Becker and Guarente, In Abelson, J.N. and Simon, M.I., editors, Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology, Volume 194, pp 182-

187, Academic Press, Inc., New York; Ito et al., 1983, Journal of Bacteriology 153:163; or Hinnen et al., 1978, Proceedings of the National Academy of Sciences USA 75:1920.

Examples of filamentous fungal cells include filamentous forms of the subdivision *Eumycota* and *Oomycota* (as defined by Hawksworth et al., 1995, supra), in particular it may a cell of a species of *Acremonium*, such as *A. chrysogenum*, or *Aspergillus*, such as *A. awamori*, *A. foetidus*, *A. japonicus*, *A. niger*, *A. nidulans* or *A. oryzae*, or Fusarium, such as *F. bactridioides*, *F. cerealis*, *F. crookwellense*, *F. culmorum*, *F. graminearum*, *F. graminum*, *F. heterosporum*, *F. negundi*, *F. reticulatum*, *F. roseum*, *F. sambucinum*, *F. sarcochroum*, *F. sulphureum*, *F. trichothecioides* or *F. oxysporum*, *Humicola*, such as *H. insolens* or *H. lanuginose*, or *Mucor*, such as *M. miehei*, or *Myceliophthora*, such as *M. thermophilum*, or *Neurospora*, such as *N. crassa*, or *Penicillium*, such as *P. purpurogenum*, or *Thielavia*, such as *T. terrestris*, or *Tolypocladium*, or *Trichoderma*, such as *T. harzianum*, *T. koningii*, *T. longibrachiatum*, *T. reesei* or *T. viride*, or a teleomorph or synonym thereof. The use of *Aspergillus spp.* for the expression of proteins is described in, e.g., EP 272 277, EP 230 023.

Examples of insect cells include a *Lepidoptera* cell line, such as *Spodoptera frugiperda* cells or *Trichoplusia ni* cells (cf. US 5,077,214). Culture conditions may suitably be as described in WO 89/01029 or WO 89/01028.Transformation of insect cells and production of heterologous polypeptides therein may be performed as described in US 4,745,051; US 4, 775, 624; US 4,879,236; US 5,155,037; US 5,162,222; EP 397,485.

Examples of mammalian cells include Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, COS cells, or any number of other immortalized cell lines available, e.g., from the American Type Culture Collection. Methods of transfecting mammalian cells and expressing nucleic acid sequences introduced in the cells are described in e.g. Kaufman and Sharp, J. Mol. Biol. 159 (1982), 601 - 621; Southern and Berg, J. Mol. Appl. Genet. 1 (1982), 327 - 341; Loyter et al., Proc. Natl. Acad. Sci. USA 79 (1982), 422 - 426; Wigler et al., Cell 14 (1978), 725; Corsaro and Pearson, Somatic Cell Genetics 7 (1981), 603, Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Inc., N.Y., 1987, Hawley-Nelson et al., Focus 15 (1993), 73; Ciccarone et al., Focus 15 (1993), 80; Graham and van der Eb, Virology 52 (1973), 456; and Neumann et al., EMBO J. 1 (1982), 841 - 845. Methods for transfecting mammalian cells are well known to a person skilled in the art and include transfection by direct uptake using the calcium phosphate precipitation method of Graham and Van der Eb (1978, Virology 52:546).

Cultivation of host cells

In the method of the present invention the host cell is cultivated on a solid media. In the context of the present invention the term "solid media" refers to the basic state of matter as a solid. In the context of solid media for cultivating host cells these include to media which are solidi-

fied using a solidifying agent such as agar. Other examples of compounds which may be used a solid media include, but are not limited to agar, agarose (?), pectate, pectin or gelatine, however this is not an exhaustive list and other examples of solid media are well-known to a person skilled in the art of culturing cells.

In one embodiment of the present invention the host cell may be cultured on a conventional plate, e.g. a conventional agar plate or a plate comprising another solid media or a plate comprising a combination of different solid media.

In another embodiment of the present invention the host cell of the present invention may be cultivated in a bead of solid media, e.g. a bead of agar or another solid media such as one of those described above. For example the host cells of the present invention may be encapsulated in a small sphere in a way that allows each cell to multiply and form a colony within its respective sphere or bead. In this way the individual host cells are compartmentalisation. Methods for performing the encapsulation within beads of appropriate size and homogeneity have been developed (Nir et al., Appl. Environ. Microbiol. 56:2870-2875, 1990). This example is based on low-melting-agarose as the gelling/solidifying agent but is not limited to this, other gelling agents such as guar gum, pectate/pectin may also be used.

Detection of a change in colour

Detection of a change in colour, i.e. the conversion of the first dye into the second dye may be detected by any means, e.g. visually or automatic. The method of choice typically depends on how the host cell has been cultivated.

For example if the host cell has been cultivated on a conventional agar plate expression of an enzyme of interest may generally be detected by the presence of a zone of halo surrounding the host cell which is of a different colour than the rest of the solid media, as the presence of the enzyme of interest result, as described above, in a conversion of the first dye into a second dye which is of a different colour than the first dye. The presence of such zones or halos surrounding a host cell expressing an enzyme of interest may then be used to identify host cells expressing an enzyme of interest. Said identification of the zones or halos may be performed by visual or automatic inspection of the agar plate comprising the host cell.

The contrast between the colour of the first and the second dye may be enhanced by using higher amounts (e.g. higher concentration) of the first dye or as described above by the presence of a polymer capable of binding the second dye and thereby reduce diffusion of the second dye in the solid media.

If the host cell has been cultivated in a bead of solid media, then the presence of host cells expressing an enzyme of interest in a bead may generally cause said bead to change to a different colour than the other beads, as the presence of the enzyme of interest results in a conversion of the first dye to a second. Identification of beads with a changed colour may be per-

formed visually or in particular it may be performed automatically by e.g. the use of a flow cytometer, such as a FACS (Fluorescence-Activated Cell Sorter).

MATERIALS AND METHODS

Materials

Enzymes

HRP (Horseradish Peroxidase): #P8125, Sigma-Aldrich, which is a peroxidase obtained from Horseradish

GOX (Glucose Oxidase): #G6125, Sigma-Aldrich, which is a glucose oxidase obtained from *Aspergillus niger*

SC-Blue-Pox-agar.

In 10L container mix:

Agar (#101614, Merck)	200g
Yeast Nitrogen W/O aminoacids (#51484, FLUKA)	75g
Succinic acid (#S7501, Sigma-Aldrich)	113g
NaOH (#6498, Merck)	68g
Casamino acids (#0288, DIFCO)	56g
L-Tryptophan (#8374, Merck)	1g
MilliPore H2O	to 8000mL

Sterilize by autoclavation.

Mix to total 10L

After sterilizing the agar was kept at 60°C and the following was added: 800mL 50% Fructose and 100mL 50% Maltose (maltose and fructose are sterile by filtartion through 0,2 microm sterilefilter both solutions were heated to 60°C before use).

Thereafter 200 mL of 60°C MilliPore water containing 4 g Remazol Brilliantblue (#R8001 Sigma-Aldrich) was added and the solution was sterile-filtered.

Then 1000 mL of 60°C Millipore water with 50 g CMC (Sodium carboxymethylcellulose, #21900, FLUKA, Sigma-Aldrich) was added. The CMC solution was heated on a magnetic stirrer until it was solubilised, before use the CMC was autoclaved.

Just before pouring plates the following was added:

240 microL HRP (5000 U/mL Horseradish peroxidase (HRP) (Dilution of #P8125, Sigma-Aldrich),

5 mL GOX (2500 U/mL Glucose Oxidase (GOX) (Dilution of #G6125, Sigma-Aldrich) and

4 mL AMP 250mg/mL Ampicillin (#A9518, Sigma-Aldrich)

Examples

Example 1

A derivative of the yeast *Saccharomyces cerevisiae* ATCC 26109 strain (the derivative has been disrupted in the Ura3 gene using a 5-FOA selection, making it Uracil dependent) was transformed with a plasmid containing the gene of the *Talaromyces emersonii* amyloglycosidase (AMG) under transcriptional control of the Triose Phosphate Isomerase promoter. The plasmid is a derivative of pYES2 (Invitrogen) and encodes a 2my origen of replication for propagation in yeast and the Ura3 gene (for positive selection of plasmid containing yeast clones on Uracil free plates). Furthermore the plasmid contains *E.coli* origen of replication and ampicillin resistance gene derived from pUC19 plasmid. (The plasmid was constructed essentially as described in the Material and Methods section of patent WO200104273). The signal peptide of the *Talaromyces emersonii* AMG directs the enzyme to the exterior of the cell.

The transformed cells were plated onto a SC-Blue-Pox-agar-plate (see recipe below)).

One positive clone positively expressing AMG was identified and kept as the positive control strain: AMG+.

The positive expression of AMG from this strain was verified by incubating the yeast strain in 10 ml YPD media at 30°C for 4 days and testing the supernatant in the AMG assay essentially as described in the Material and Methods section of patent WO200104273).

A negative control of no AMG expression was the Uracil dependent the yeast *Saccharomyces cerevisiae* ATCC 26109 strain. Whenever this strain (called AMG-) was tested, Uracil was included in liquid and solid media (final concentration of 20 mg/l of Uracil).

AMG+ and AMG- strains were suspended in water and a small amount of further diluted cells was spread out on SC-Blue-Pox-agar-plate (with and without Uracil respectively for each strain) after 2-3 days incubation at 30 degrees Celsius. AMG- strain had no orange clearing zones around colonies and AMG+ strain expressing AMG were detected visually by the presence of a clear orange halo surrounding the clone as the following reaction between the AMG and the compounds (maltose, Glucose Oxidase, Horseradish Peroxidase and Brilliant Blue) present in the agar plate takes place:

AMG + maltose → glucose + Glucose Oxidase → H_2O_2 + Horseradish Peroxidase + Brilliant Blue → Orange compound

Results:

After incubation for 2-3 days orange halos were present around colonies of the AMG+ strain and not around the AMG- strain, which indicated that AMG+ colonies expressed active AMG.

No orange halos could be seen around AMG- colonies even after 7 days of incubation.

CLAIMS

1. A method for testing or screening a host cell for expression of an enzyme of interest, comprising testing or screening for a change in the colour of a first dye by the presence of a host cell expressing the enzyme of interest, wherein the host cell has been cultivated on or in a solid media in the presence of a first substrate, one or more other enzymes and a first dye, and wherein a product of the chemical reaction between the enzyme of interest and the first substrate is a substrate for one of the other enzymes, and wherein the first dye is a substrate for one of the other enzymes, and wherein the product of the chemical reaction between the first dye and one of the other enzyme is a second dye, and wherein the colour of the first dye is different from the colour of the second dye.

2. A method according to claim 1, wherein the method comprises the following steps:

c) cultivating a host cell expressing the enzyme of interest on or in a solid media in the presence of a substrate for the enzyme, one or more other enzymes and a dye, wherein the product of the reaction between the substrate and the enzyme is a substrate for one of the other enzymes, and wherein the dye is a substrate for one of the other enzymes, and wherein the product of the reaction between one of the other enzymes and the dye has a different colour than the compound.

d) identifying a host cell by the presence of the colour of the second dye.

3. A method according to any of the preceding claims, wherein the host cells are also cultivated in the presence of a polymer capable of binding the second dye.

4. A method according to claim 3, wherein the polymer is carboxy methyl cellulose (CMC), chitin, chitosan, pectate, pectin or starch.

5. A method according to any of the preceding claims, wherein the other enzymes comprise a peroxidase (E.C. 1.11.1.7).

6. A method according to claim 5, wherein the other enzymes further comprise an enzyme capable of producing hydrogen peroxide upon reaction with its substrate, e.g. a glucose oxidase (E.C. 1.1.3.4), a cellobiose oxidase (E.C. 1.1.3.25), an alcohol oxidase (E.C. 1.1.3.13), a galactose oxidase (E.C. 1.1.3.9) or a L-amino acid oxidase (E.C.1.4.3.2).

7. A method according to any of claims 1-6, wherein the enzyme of interest is selected from the group consisting of: a glucoamylase (E.C. 3.2.1.3), a beta-glucosidase (E.C. 3.2.1.21), a pectinesterase (E.C. 3.1.1.11), a alpha-galactosidase (E.C. 3.2.1.22), a cellulose 1,4-beta-cellobiosidase (E.C. 3.2.1.91), a lactase (E.C.3.2.1.108), a beta-galactofuranosidase and a carboxypeptidase A (E.C. 3.4.17.1).

8. A method according to claim 6, wherein the other enzymes further comprise a beta-glucosidase (E.C. 3.2.1.21).

9. A method according to any of claims 1-6 or claim 8, wherein the enzyme of interest is a cellulase (E.C.3.2.1.4) or a cellulose 1,4-beta-cellobiosidase (E.C. 3.2.1.91).

10. A method according to any of claims 1-5, wherein the enzyme of interest is an enzyme for which a product of the chemical reaction between the enzyme of interest and a first substrate is hydrogen peroxide.

11. A method according to claim 10, wherein the enzyme of interest is selected from the group consisting of: a glucose oxidase (E.C. 1.1.3.4), a cellobiose oxidase (E.C. 1.1.3.25), an alcohol oxidase (E.C. 1.1.3.13), a galactose oxidase (E.C. 1.1.3.9) and a L-amino acid oxidase (E.C.1.4.3.2).

12. A method according to any of the preceding claims, wherein the first dye is Brilliant Blue.

13. A method for testing or screening for an activity of an enzyme of interest comprising testing of screening for a change in a colour of a first dye by the presence of a host cell expressing an enzyme of interest, wherein the host cell has been cultivated on a solid media in the presence of a first dye and a polymer, wherein the product of the chemical reaction between the first dye and the enzyme of interest is a second dye, and wherein the polymer is capable of binding to the second dye, and wherein the colour of the first dye is different from the colour of the second dye.

14. A method according to claim 13, wherein the method comprises the following steps:

a) cultivating a host cell expressing the enzyme on a solid media in the presence of a first dye and a polymer, wherein the product of the chemical reaction between the first dye and the enzyme of interest is a second dye, and wherein the polymer is

capable of binding to the second dye, and wherein the colour of the first dye is different from the colour of the second dye.

b) identifying a host cell by the presence of the colour of the second dye.

15. A method according to any of claims 13-14, wherein the polymer is carboxy methyl cellulose (CMC), chitin, chitosan, pectate, pectin or starch.

16. A method according to any of claims 13-15, wherein the first dye is Brilliant Blue.

17. A method according to any of claims 13-16, wherein the enzyme of interest is a peroxidase (E.C. 1.11.1.7), e.g. Horseradish peroxidase.

18. A method for testing or screening for an activity of a peroxidase (E.C. 1.11.1.7) comprising testing of screening for a change in the colour of Brilliant Blue by the presence of a host cell expressing the peroxidase, wherein the host cell has been cultivated on a solid media in the presence of Brilliant Blue and hydrogen peroxide.

ABSTRACT

The present invention relates to a method for testing or screening for an enzyme of interest.